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Hypermethylation-associated Inactivation of *p14^{ARF}* Is Independent of *p16^{INK4a}* Methylation and *p53* Mutational Status[1]

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ABSTRACT

The INK4a/ARF locus encodes two cell cycle-regulatory proteins, *p16^{INK4a}* and *p14^{ARF}*, which share an exon using different reading frames. *p14^{ARF}* antagonizes *MDM2*-dependent *p53* degradation. However, no point mutations in *p14^{ARF}* not altering *p16^{INK4a}* have been described in primary tumors. We report that *p14^{ARF}* is epigenetically inactivated in several colorectal cell lines, and its expression is restored by treatment with demethylating agents. In primary colorectal carcinomas, *p14^{ARF}* promoter hypermethylation was found in 31 of 110 (28%) of the tumors and observed in 13 of 41 (32%) colorectal adenomas but was not present in any normal tissues. *p14^{ARF}* methylation appears in the context of an adjacent unmethylated *p16^{INK4a}* promoter in 16 of 31 (52%) of the carcinomas methylated at *p14^{ARF}*. Although *p14^{ARF}* hypermethylation was slightly overrepresented in tumors with wild-type *p53* compared to tumors harboring *p53* mutations [19 of 55 (34%) *versus* 12 of 55 (22%)], this difference did not reach statistical significance. *p14^{ARF}* aberrant methylation was not related to the presence of K-*ras* mutations. Our results demonstrate that *p14^{ARF}* promoter hypermethylation is frequent in colorectal cancer and occurs independently of the *p16^{INK4a}* methylation status and only marginally in relation to the *p53* mutational status.

INTRODUCTION

Disruption of the *p53* and *Rb*[3] tumor suppressor pathways is a fundamental trend of most human cancer cells (1). In tumorigenesis, loss of *Rb* function can occur by direct inactivation of the *Rb* gene itself through mutation, sequestration of the *Rb* protein by viral oncoproteins, or promoter hypermethylation or by deregulation of the genes controlling Rb phosphorylation status (2). These last alterations include cyclin D1 gene amplification, *CDK4* activating mutations, and also gene amplification and inactivation of the inhibitors of *CDK4*, the *INK4* family (composed of *p15^{INK4b}*, *p16^{INK4a}*, *p18^{INK4c}*, and *p19^{INK4d}*; Refs. 1 and 3). The *p16^{INK4a}* gene was implicated as a tumor suppressor gene by its frequent mutation, deletion, or promoter hypermethylation in a variety of human tumors (1, 4, 5). In addition, *p16^{INK4a}* germ-line mutations have been associated with familial melanoma (6). Recently, it was demonstrated that a portion of the *p16^{INK4a}* gene has the capacity to encode a second product, murine *p19^{ARF}* (7). *p19^{ARF}*, or human *p14^{ARF}*, has a unique first exon (denominated exon 1β), located approximately 20 kb centromeric to the first exon of *p16^{INK4a}* (denoted as exon 1α). Under the control of its own promoter, exon 1β splices into exon 2 of INK4a in an alternative reading frame, producing a different protein than *p16^{INK4a}* (1, 3, 7, 8).

p19^{ARF} overexpression induces G_1- and G_2-phase arrest through a *p16^{INK4a}*-independent mechanism (9). Furthermore, the *p19^{ARF}*-mediated cell cycle arrest seems to be abolished in mouse embryo fibroblasts lacking functional *p53* (8). Recent work suggests that *p19^{ARF}* and *p14^{ARF}* interact *in vivo* with the MDM2 protein, neutralizing *MDM2*-mediated degradation of *p53* (10–13). Thus, theoretically, inactivation of *p14^{ARF}* would then be predicted to decrease the frequency for concomitant *p53* mutations. *p19^{ARF}* binds to *MDM2* through its NH_2 terminus end, which encodes exon 1β (13). *p19^{ARF}*-specific null mice carrying a disrupted exon 1β are cancer prone at an early age (8), but no human point mutations in exon 1β have been reported. Interestingly, exon 1β-specific deletions have been described in melanoma cell lines (14), and *p14^{ARF}* genomic alterations are found in a majority of T-cell acute lymphocytic leukemias (15). However, to date, most of the functional studies of *p14^{ARF}* have involved murine systems, and little is known about the putative *p14^{ARF}* function as a tumor suppressor gene in humans. Recently, the human *p14^{ARF}* promoter has been cloned and contains a CpG island that is aberrantly methylated in colorectal cancer cell lines (16). Hypermethylation of normally unmethylated CpG islands in the promoter regions of tumor suppressor and DNA repair genes, including *p16^{INK4a}*, *E-cadherin*, *hMLH1*, *GSTP1*, and *MGMT* (4, 17–20), correlates with loss of transcription. Thus, promoter hypermethylation could be a mechanism for *p14^{ARF}* inactivation in human tumors. In addition, methylation of *p16^{INK4a}* and *p15^{INK4b}* can occur with more tumor-specific patterns than found for homozygous deletions involving the entire 9p region encompassing these genes (21).

In the present study, we studied CpG island promoter methylation of the human *p14^{ARF}* gene in cancer cell lines and more than 100 primary colorectal carcinomas. Colorectal tumors were chosen because homozygous deletions of the INK4a/ARF locus are not present in this tumor type (22), and colorectal carcinoma has well-characterized mutational inactivation of *p53*, thus allowing us to examine the relationship to p53 mutations. Our results demonstrate that *p14^{ARF}* can be silenced by promoter hypermethylation in colorectal cancer cell lines. In the primary colorectal tumors, *p14^{ARF}* is aberrantly methylated in approximately a quarter of the neoplasms studied, and this methylation represents an event independent of the *p16^{INK4a}* methylation status. Furthermore, *p14^{ARF}* hypermethylation, although more frequent in tumors with wild-type *p53*, is also observed in those cancers with *p53* mutations.

MATERIALS AND METHODS

MSP. DNA methylation patterns in the CpG islands of the *p14^{ARF}* gene were determined by MSP (23). MSP distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated (but not methylated) cytosines to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA (23). The primer sequences designed for *p14^{ARF}* spanned six CpGs within the 5′ region of the gene. Primer sequences of *p14^{ARF}* for the unmethylated reaction were 5′-TTTTTGGTGTTA-

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[3] The abbreviations used are: Rb, retinoblastoma; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

AAGGGTGGTGTAGT-3′ (sense) and 5′-CACAAAAACCCTCACTCA-CAACAA-3′ (antisense), which amplify a 132-bp product, and primer sequences of $p14^{ARF}$ for the methylated reaction were 5′-GTGTTAAAGGGCGGCG-TAGC-3′ (sense) and 5′-AAAACCCTCACTCGCGACGA-3′ (antisense), which amplify a 122-bp product. The 5′ position of the sense unmethylated and methylated primers corresponds to bp 195 and 201 of GenBank sequence number L41934. Both antisense primers originate from bp 303 of this sequence. The annealing temperature for both the unmethylated and methylated reactions was 60°C. Placental DNA treated *in vitro* with *Sss*I methyltransferase was used as a positive control for methylated alleles. DNA from normal lymphocytes was used as a negative control for methylated genes. Ten μl of each PCR reaction were loaded directly onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. DNA methylation patterns in the 5′-CpG island of $p16^{INK4a}$ were determined by MSP as described previously (23).

RT-PCR. RT-PCR was performed as described previously (21), using 3 μg of total cellular RNA to generate cDNA. This cDNA (100 ng) was amplified by PCR with primers for exon 1β (5′-GGTTTTCGTGGTTCACATCCCGCG-3′) and exon 2 (5′-CAGGAAGCCCTCCCGGGCAGC-3′) of $p14^{ARF}$, which amplify a 254-bp product spanning sequence 204–437 from GenBank accession number S78535. RT-PCR for GAPDH served as a positive control. Ten μl of each PCR reaction were loaded directly onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Detection of K-*ras* and *p53* Mutations. Mutations at codons 12 and 13 of the K-*ras* gene were detected and characterized by a RFLP/PCR approach (24). *p53* mutations in exons 4–9 were analyzed by single-strand conformational polymorphism analysis. Briefly, a first PCR was performed using primers 12979U (GCTGCCGTGTTCCAGTTGCT) and 14875D (AGGCATCACT-GCCCCCTGAT). The resulting 1897-bp fragment was then used as a template to separately amplify a fragment of 410 bp including exons 5 and 6 [with primers 13054U (TACTCCCCTGCCCTCAACAAG) and 13463D (CTCCTC-CCAGAGACCCCAGT)] and a fragment of 622 bp including exons 7 and 8 [with primers 13966U (CTGGCCTCATCTTGGGCCTG) and 14587D (CTCGCTTAGTGCTCCCTGGG)]. These two fragments were then digested with restriction enzyme *Hpa*II, and the resulting fragments were run on a 6% polyacrylamide gel without glycerol (0.2 h at 30 W and 5–6 h at 6 W) and with 10% glycerol (0.2 h at 30 W and 13–14 h at 6 W) to detect mobility shifts. Mutations were confirmed by direct cycle sequencing of the PCR products using the AmpliCycle Sequencing Kit (Perkin-Elmer, Branchburg, NJ). Exons 4 and 9 were only analyzed on those samples negative for mutations in exons 5–8. Exon 4 was amplified directly from DNA using primers 12019U (GTC-CCCCTTGCCGTCCCAAG) and 12349D (TACGGCCAGGCATTGAA-GTC). The resulting 331-bp fragment was run without previous digestion on a 6% polyacrylamide/10% glycerol gel for 0.2 h at 30 W and 19 h at 6 W. To analyze exon 9, a fragment of 788 bp including exons 7–9 was amplified with primers 13966U (CTGGCCTCATCTTGGGCCTG) and 14753D (CTGAAG-GGTGAAATATTCTCC) and digested with *Hha*I to produce two fragments of 548 and 240 bp, the latter of which contained exon 9.

RESULTS

Promoter Hypermethylation and Expression of $p14^{ARF}$ in Cancer Cell Lines. Structurally, $p14^{ARF}$ is a candidate for hypermethylation-associated inactivation because a 5′-CpG island is located around the transcription start site (16). The region chosen for the MSP analysis spans the area of greatest CpG density studied recently for methylation changes in several cell lines (16). Normal lymphocytes, colon, breast, endometrium, and lung were found completely unmethylated at the $p14^{ARF}$ promoter (Fig. 1*A*). Normal kidney and liver were also unmethylated at $p14^{ARF}$. We also examined 16 cancer cell lines derived from these tissues. $p14^{ARF}$ was fully methylated in four (25%) colorectal carcinoma cell lines (SW48, RKO, DLD-1, and LoVo; Fig. 1*B*). Among these, expression of the $p14^{ARF}$ transcript by RT-PCR was assessed in LoVo and DLD-1, both of which lack $p14^{ARF}$ expression (Fig. 1*C*). Treatment of these cell lines with the demethylating agent 5-aza-2′-deoxycytidine restored the expression of the transcript in both cases (Fig. 1*C*). These results agree with those recently reported by Robertson *et al.* (16), although in that case, the colorectal cancer cell line HCT-15, which is isogenic to the DLD-1 cell line (25), was used. The colorectal cancer cell line RKO methylated at $p14^{ARF}$ showed a low level of expression but demonstrated an increase in RNA after the treatment with 5-aza-2′-deoxycytidine. A similar phenomenon has been described previously in the case of the colorectal cancer cell line SW48 (16), which was also methylated at $p14^{ARF}$ in our study. The colorectal cancer cell lines SW1417, SK-CO1, LS180T, and HT-29 were partially methylated, and $p14^{ARF}$ expression was demonstrated in the last one (Fig. 1*C*). Normal lymphocytes and the unmethylated colorectal cell line SW480 and the unmethylated leukemia cell line HL-60 demonstrated expression of the $p14^{ARF}$ transcript (Fig. 1*C*).

The proposed role of $p14^{ARF}$ in modulating *p53-MDM2* function would predict the diminishing need of *p53* mutations in those tumors or cell lines with $p14^{ARF}$ promoter hypermethylation, assuming that only one "hit" in the same pathway would be necessary in the transforming process. In this series of cancer cell lines, cell lines fully methylated at $p14^{ARF}$ (LoVo, DLD-1, SW48, and RKO) were more frequently found to have an intact *p53* [three of four cell lines (75%), with the exception DLD-1]. Among cell lines with an unmethylated promoter, including colorectal cells, non-small cell lung cancer, and leukemia (SW480, HCT116, SW837, COLO205, H157, H1618, U1752, and HL-60), only one (HCT116) had a wild-type *p53*. Thus, most cancer cell lines methylated at $p14^{ARF}$ are wild-type *p53*, and those unmethylated have a mutant *p53*, but this difference did not reach statistical significance (Fisher's exact test, $P = 0.07$).

Promoter Hypermethylation of $p14^{ARF}$ in Primary Colorectal Carcinomas. DNA obtained from 110 primary colorectal carcinomas was subjected to $p14^{ARF}$ promoter methylation study using MSP. Among all of the colorectal tumors studied, $p14^{ARF}$ promoter hypermethylation was present in 31 of 110 (28%) samples (Fig. 1*D*). In 32 patients from whom normal adjacent mucosa DNA was available, no methylation of the $p14^{ARF}$ promoter was observed in any case (an example is shown in Fig. 1*A*). Thus, the methylation observed in the colorectal cancers and cell lines is a tumor-specific change. Abnormal methylation of the $p14^{ARF}$ promoter region in the colorectal carcinomas was not associated with significant differences of gender, age of onset, clinical status, Duke's stage, DNA ploidy, or the presence of residual disease. When the samples were decoded for their *p53* status, $p14^{ARF}$ was hypermethylated in 19 of 55 (34%) colorectal tumors with wild-type *p53* and in 12 of 55 (22%) tumors with a mutant *p53* (Fig. 2*A*). Thus, although the tumors with functional *p53* more often harbor $p14^{ARF}$ epigenetic inactivation than tumors with p53 mutations, this trend does not reach statistical significance (Fisher's exact test, $P = 0.20$) and was certainly not limited to *p53* wild-type tumors (Fig. 2*A*).

We also wondered whether $p14^{ARF}$ promoter hypermethylation would be related to promoter hypermethylation of the adjacent gene, $p16^{INK4a}$. Aberrant methylation of $p16^{INK4a}$ was assessed in the same samples analyzed for $p14^{ARF}$ methylation and demonstrated in 41 of 110 (37%) of the primary colorectal carcinomas studied, a frequency similar to that described previously (Ref. 26; Fig. 1*D*). $p14^{ARF}$ was hypermethylated in the presence of an unmethylated $p16^{INK4a}$ promoter in 16 of 110 (14%) of the cases, whereas $p14^{ARF}$ promoter hypermethylation was coincident with $p16^{INK4a}$ promoter hypermethylation in 15 of 110 (14%) of the cases (Fig. 2*B*). In 26 of 110 (24%) of the cases, $p16^{INK4a}$ was methylated without $p14^{ARF}$ methylation (Fig. 2*B*). Thus, methylation at the $p14^{ARF}$ and $p16^{INK4a}$ promoters does not seem to be directly related (Fisher's exact test, $P = 0.28$). It has been hypothesized that mutations in exons 1–4 of *p53* may be concomitant with alterations in both $p16^{INK4a}$ and $p14^{ARF}$

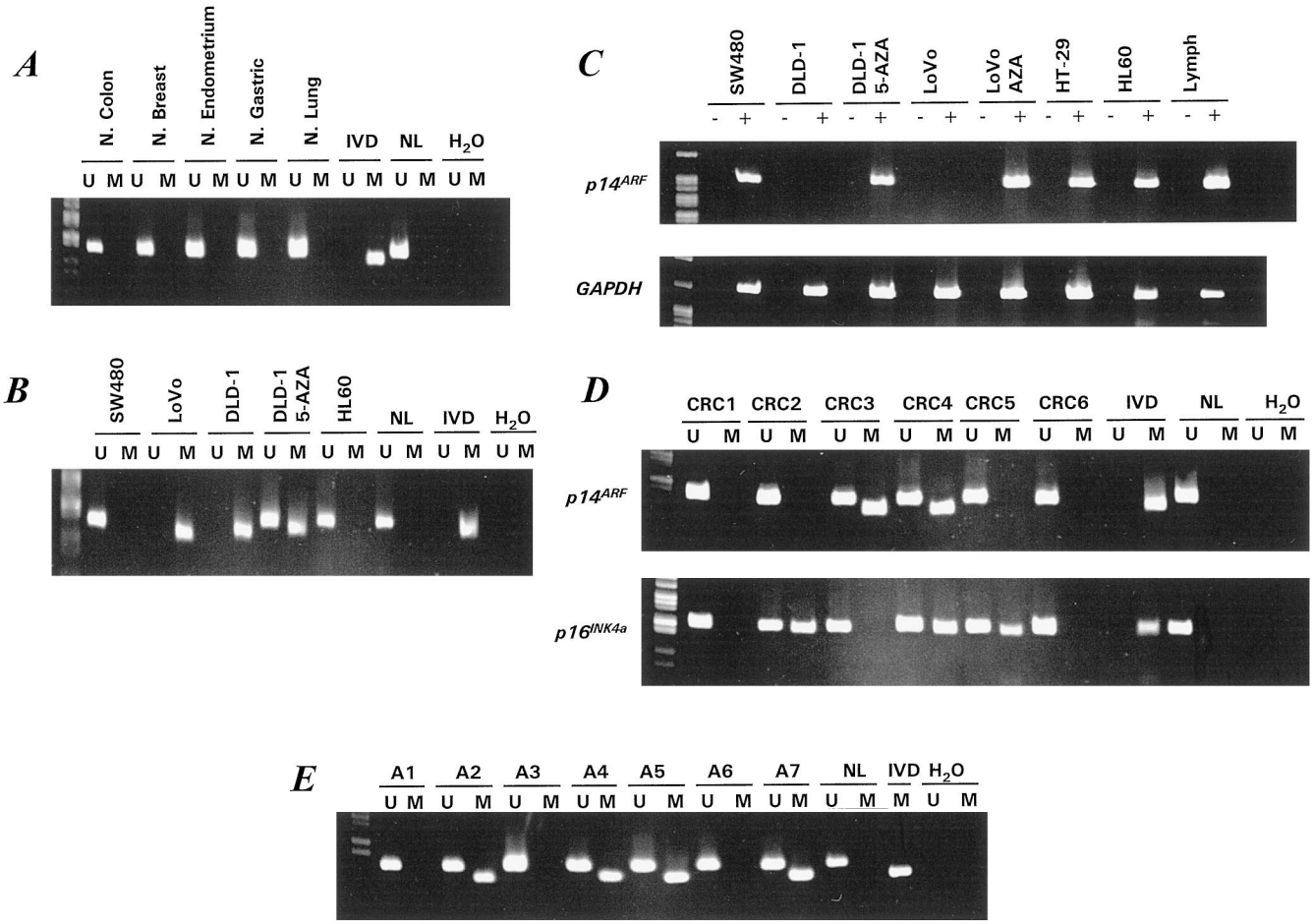

Fig. 1. MSP analysis of the promoter region of $p14^{ARF}$ and reactivation of $p14^{ARF}$ by treatment with 5′-aza-2′-deoxycytidine. The presence of a visible PCR product in *Lanes U* indicates the presence of unmethylated genes of $p14^{ARF}$; the presence of product in *Lanes M* indicates the presence of methylated genes. *In vitro* methylated DNA (*IVD*) was used as a positive control for $p14^{ARF}$ promoter hypermethylation, and normal lymphocytes (*NL*) were used as a negative control for methylation. Water controls for PCR reactions are also shown. MSP of $p14^{ARF}$ in normal tissues (*A*) and cancer cell lines (*B*). *C*, the pattern of expression determined by RT-PCR of the $p14^{ARF}$ transcript in cancer cell lines SW480, DLD-1, DLD-1 after 5-aza-2′-deoxycytidine treatment, LoVo, LoVo after 5-aza-2′-deoxycytidine treatment, HT-29, and HL-60 and in normal lymphocytes. GAPDH expression demonstrates relatively equal amounts of initial mRNA. *D*, MSP of $p14^{ARF}$ in primary colorectal carcinomas (*CRC*) of $p14^{ARF}$ (*top panel*) and $p16^{INK4a}$ (*bottom panel*). CRC1 and CRC6 are unmethylated at both genes, CRC2 and CRC5 are methylated only at $p16^{INK4a}$, CRC3 is methylated only at $p14^{ARF}$, and CRC4 is methylated at both genes. *E*, MSP of $p14^{ARF}$ in colorectal adenomas. $p14^{ARF}$ promoter hypermethylation is demonstrated in samples A2, A4, A5, and A7.

(27). Although we did not address this aspect specifically, in the three cases in which exon 4 mutations were found, $p16^{INK4a}$ was unmethylated in all three, and $p14^{ARF}$ was methylated in only one case.

Because it has been recently reported that $p19^{ARF}$ (the mouse homologue of $p14^{ARF}$) is essential for the activation of *p53* in response to oncogenic *Ras* (28), we also examined the $p14^{ARF}$ promoter hypermethylation in relation to the K-*ras* status of the tumor. A total of 43 of 110 (39%) primary colorectal carcinomas had a K-*ras* point mutation. The frequency of $p14^{ARF}$ methylation was slightly higher in tumors with K-ras mutations than in those without K-ras mutations [15 of 43 (35%) *versus* 16 of 67 (24%)], but this difference was not statistically significant (Fisher's exact test, $P = 0.28$). Thus, no evident linkage between these epigenetic and genetic alterations was observed.

Promoter Hypermethylation and Expression of $p14^{ARF}$ in Colorectal Adenomas. DNA obtained from 41 primary colorectal adenomas was subjected to $p14^{ARF}$ promoter methylation study using MSP. Among all of the adenomas studied, $p14^{ARF}$ promoter hypermethylation was present in 13 of 41 (32%) samples (Fig. 1*E*). We also examined the expression of $p14^{ARF}$ using RT-PCR in these colorectal adenomas. Among 20 early lesions with available cDNA, 12 colorectal adenomas unmethylated at $p14^{ARF}$ expressed high levels of $p14^{ARF}$ mRNA, whereas 8 adenomas with $p14^{ARF}$ methylation expressed no detectable $p14^{ARF}$ mRNA ($n = 7$) or very little $p14^{ARF}$ mRNA ($n = 1$), demonstrating an exact correlation of transcriptional loss with $p14^{ARF}$ hypermethylation (Fig. 3). The similar rate of $p14^{ARF}$ methylation in adenomas and carcinomas suggests that like $p16^{INK4a}$ methylation, inactivation of $p14^{ARF}$ appears to be an early event in colorectal tumorigenesis.

DISCUSSION

Our study represents the first finding of a specific lesion in $p14^{ARF}$, promoter hypermethylation, in primary human tumors. In many cases, this does not affect the neighboring gene, $p16^{INK4a}$. Furthermore, our data establish that, at least in colorectal tumors, the inactivation on $p14^{ARF}$ is not restricted to those neoplasms with intact *p53*. It has been postulated that due to the fact that *p53* is directly targeted in approximately 50% of human malignancies (29), other mechanisms are required to shut down the *p53* pathway in tumors with wild-type *p53*. For example, lesions in the *MDM2* gene such as amplification can lead to *MDM2* overexpression and abrogation of *p53* function. *MDM2* represses *p53* transcriptional activity and mediates the degradation of *p53* (30). However, *MDM2* gene amplification is restricted to a few tumor types (30). *p53* degradation mediated by the viral protein E6 is also limited to a minority of neoplasms (31). Another event contrib-

uting to abrogate the *p53* pathway in the cancer cell may be $p14^{ARF}$ loss of function. Somatic mutations affecting exons 2 and 3 $p14^{ARF}$, shared in an alternative reading frame with $p16^{INK4a}$, have been demonstrated in tumors (1, 5), but no mutations in the specific exon 1β of $p14^{ARF}$ have been reported. $p14^{ARF}$ can also be lost by homozygous deletion in several tumor types (22), but this loss also targets $p16^{INK4a}$ in the vast majority of cases. However, $p14^{ARF}$ has been proposed as the crucial target in T-cell acute lymphocytic leukemias and glioblastomas that exhibit 9p21 lesions (15). Recently, aberrant methylation of the $p14^{ARF}$ promoter has been demonstrated in cancer cell lines (16). Our data corroborate the findings of loss of $p14^{ARF}$ expression associated with CpG island methylation and its restoration by the use of demethylating agents. We now demonstrate that such hypermethylation of $p14^{ARF}$ is not limited to cell lines and is not a rare event. Colorectal carcinomas were selected because these cancers do not show homozygous deletion of the 9p21 locus (22) and thus allow the separate study of $p14^{ARF}$ and $p16^{INK4a}$ inactivation. In addition, the considerable rate of *p53* mutations in colorectal tumors helps us to address the existence or nonexistence of a relation between $p14^{ARF}$ and *p53* status.

Our results demonstrate that $p14^{ARF}$ promoter hypermethylation occurs in approximately one-fourth of primary colorectal carcinomas. $p14^{ARF}$ promoter hypermethylation also seems to be an early event in colorectal tumorigenesis because, like $p16^{INK4a}$ methylation, it can be found in colon adenomas. When the $p14^{ARF}$ promoter methylation analysis is compared with the *p53* status, we observe only a slightly decreased occurrence of *p53* mutations in those primary tumors with $p14^{ARF}$ inactivation. Thus, $p14^{ARF}$ and *p53* can be inactivated simultaneously in the same tumor. Such a scenario is also observed in a mouse model, where tumors arising in $p19^{ARF}-/-$ mice can harbor *p53* mutations (8).

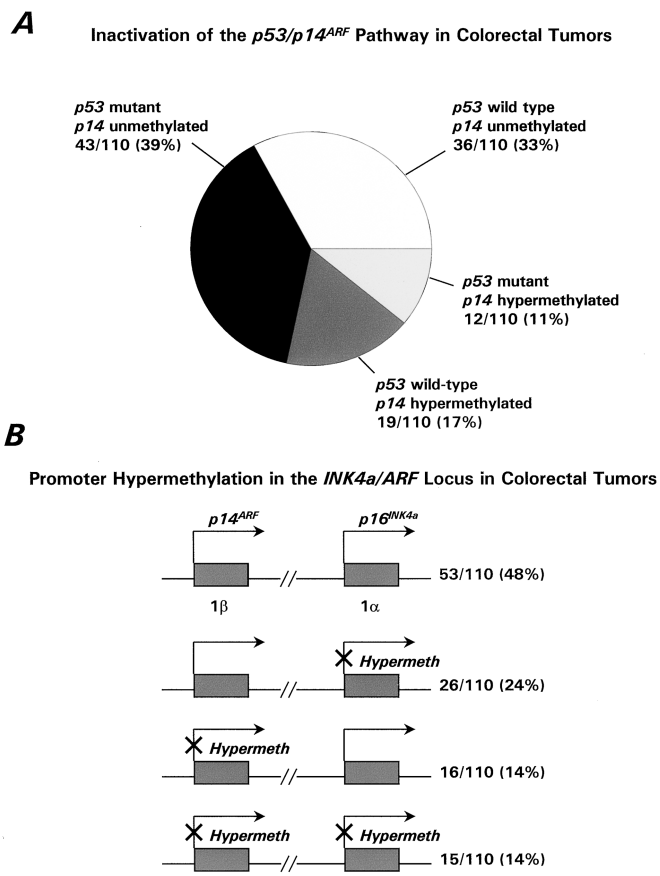

Fig. 2. Patterns of $p14^{ARF}$ promoter hypermethylation according to *p53* mutational status (*A*) and $p16^{INK4a}$ promoter hypermethylation (*B*) in colorectal tumors.

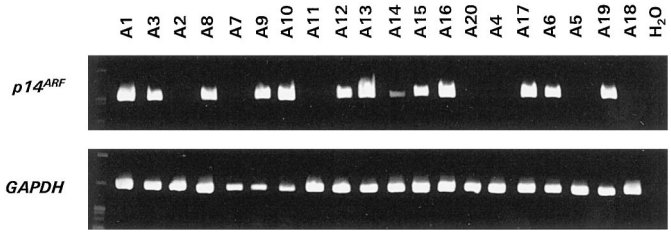

Fig. 3. Pattern of expression determined by RT-PCR of the $p14^{ARF}$ transcript in colorectal adenomas. The adenomas with $p14^{ARF}$ promoter hypermethylation show a complete lack (*A2, A7, A11, A20, A4, A5, A18*) or strongly diminished (*A14*) $p14^{ARF}$ expression. The colorectal adenomas unmethylated at $p14^{ARF}$ (*A1, A3, A8, A9, A10, A12, A13, A15, A16, A17, A6, A19*) show high levels of the transcript. GAPDH expression demonstrates relatively equal amounts of initial mRNA.

Finally, it is relevant to note that $p14^{ARF}$ promoter hypermethylation is not dependent on $p16^{INK4a}$ promoter methylation status. In our set of colorectal tumors, the most common situation is the simultaneous absence of methylation in both promoters (48%), but after this, the three possible scenarios ($p16^{INK4a}$ methylated alone, $p14^{ARF}$ methylated alone, and both methylated) are similarly represented. In primary colorectal carcinoma, hypermethylation of $p14^{ARF}$ and $p16^{INK4a}$ are independent events. In colorectal cancer cell lines, because the vast majority of cell lines are methylated at $p16^{INK4a}$, no cell line has $p14^{ARF}$ methylation with an unmethylated $p16^{INK4a}$ promoter. It is also interesting that the CpG island of $p15^{INK4b}$, which is located only 14 kb upstream of the $p14^{ARF}$ promoter, remains unmethylated in colorectal tumors and cell lines, although it is methylated in leukemia (21). Thus, the $p14^{ARF}$ promoter demonstrates selective epigenetic silencing in a subset of colorectal tumors, with a hypermethylated promoter ($p14^{ARF}$) between two unmethylated promoters ($p16^{INK4a}$ and $p15^{INK4b}$) that are frequently methylated in other tumors.

In summary, our data suggest that promoter hypermethylation of $p14^{ARF}$ is a relatively common and early event in colorectal tumorigenesis and is not necessarily related to the methylation status of neighbor gene $p16^{INK4a}$ or restricted to tumors with intact *p53*.

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Hypermethylation-associated Inactivation of *p14^{ARF}* Is Independent of *p16^{INK4a}* Methylation and *p53* Mutational Status

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